IDENTIFICATION OF A FAMILY OF ALTERNATIVELY-SPLICED ISOFORMS OF ANGIOPOIETIN-1 IN TUMOR CELLS, UPREGULATED BY THROMBIN. Y.-Q. Huang*, J.-J. Li*, S. Karpatkin. *NYU Medical School, NY, USA.*

Angiogenesis is essential for the growth and metastasis of tumors; and thrombin enhances experimental pulmonary metastasis (Blood 90:400a, 1997). We therefore looked for the presence of angiopoietin-1 (Ang-1) in tumor cells, a recently-described ligand for the endothelial specific receptor tyrosine kinase, Tie-2 required for embryonic vascular development (Cell 87:1171, 1996); and its possible regulation by thrombin. Ang-1 was detected by RT-PCR and Northern analysis in 4 of 6 cell lines studied: megakaryocyte CHRF-288, breast cancer MDA-MB468, MDA-MB231 and normal fibroblast (FS4); not in breast MCF7 or prostate DU145 cell lines. Thrombin upregulated Ang-1 mRNA in all 4 lines in which it was detected. Ang-2, an inhibitor of Ang-1, was not detected nor induced by thrombin. Surprisingly, three alternatively-spliced species of Ang-1 mRNA (Ang-1.3 Kb, 0.9 Kb and 0.7 Kb of the coding region, in addition to the full length 1.5 Kb) were identified by RT-PCR, cloned and sequenced (fifty clones examined, 8 sequenced). This was confirmed by RT-PCR using specific sense primers derived from junction sites and anti-sense primers from the 3' end of the cDNA for Ang-1. The distribution and/or expression of all 4 isoforms varied in different cells. CHRF had all 4 isoforms confirmed by Northern analysis. MD468 and FS4 had two (1.5 Kb and 1.3 Kb), MD231 had one (1.5 Kb). All 4 isoforms were upregulated and/or induced by thrombin, 0.5-1 u/ml (5-10 nM). Thrombin induction was noted as early as 30 min in FS4 cells and peaked at 3 hrs. Ang-1 contains a secretory peptide, a coiled-coil region thought to be involved in protein assembly into multimeric structures (homo or heteromultimers of related proteins) and a fibrinogen-like region. All four isoforms noted could be synthesized with a hydrophobic leader peptide for secretion, indicating their possible physiologic significance. The coiled-coil region is intact in the 1.5 Kb and 1.3 Kb isoforms, partially deleted in the 0.7 Kb isoform and mostly deleted in the 0.9 Kb isoform. We suggest that the various isoforms described in tumor cells may have different pro- or anti-angiogenic functions depending on their multimeric association and that thrombin may be critical in their regulation.

Abstract# 701 **Poster Board#/Session: 85-II**

INHIBITION OF ANGIOGENESIS BY PEPTIDES DERIVED FROM KININOGEN. R.W. Colman, Y. Lin*, D. Johnson*, S.A. Mousa*. *Sol Sherry Thrombosis Research Center, Temple University School of Medicine, Philadelphia, PA and DuPont Pharmaceuticals, Wilmington, DE, USA.*

Recent studies from our laboratory have shown that cleaved high molecular weight kininogen (HKa) binds specifically, saturably and reversibly and in a zinc-dependent fashion to the human umbilical vein endothelial cell (HUVEC) urokinase receptor (uPAR). The binding of HKa to domain 2/3 of uPAR is inhibited by vitronectin, which binds to the same site, suggesting that HKa is antiadhesive. In addition, HKa is complexed with kallikrein, which cleaves prourokinase to urokinase, which confers fibrinolytic activity on HUVEC. We therefore postulated that HKa might be proangiogenic and that peptides from HKa could complete and inhibit angiogenesis by inhibiting the interaction of HKa with uPAR. Migration of HUVEC toward vitronectin and fibronectin was measured in a Boyden chamber fitted with a chemotaxis membrane (8 microns diameter). Adherent cells were permeabilized, stained with rhodamine and the fluorescence quantified. The recombinant polypeptide, HK domain 5 (Lys420-Ser513), at a concentration of 270 nM, inhibited (85%) the migration of HUVEC to vitronectin, but inhibited less than 50% the migration to fibronectin. HUVEC cells were stimulated with basic fibroblast growth factor (b-FGF) for 48 min at 37°C under CO_2 and proliferation measured by the fluorescence enhancement of CyQUANT GR dye bound to nucleic acid of lysed cells. HK domain 5 inhibited HUVEC proliferation (100%) at 270 nM. It also blocked new vessel formation (stimulated by b-FGF) in the chicken chorioallantoic membrane. Deletion mutagenesis indicated that the domain 5 peptide Lys420-Asp474 inhibited HUVEC proliferation 94% at 360 nM. Removal of amino acids Lys420-Asp464 completely abolished the inhibition, indicating that this sequence is critical in the inhibition of HUVEC proliferation. The results suggest that polypeptides from domain 5 of HK may be potent inhibitors of angiogenesis, which could have potential for inhibiting tumor cell metastasis and invasion, diabetic retinopathy (neovascularization) and the abnormal remodeling in atherosclerosis.

Abstract# 702 **Poster Board#/Session: 86-II**

ANGIOSTATIN 4.5: A NATURALLY OCCURRING HUMAN ANGIOGENESIS INHIBITOR. Gerald A. Soff, Jerome Hong, David Fishman*, Marlon Kleinman*, Edward Kaplan*, David Zagzag*, Ryan Schultz*, Deborah Cundiff*, Susan Park*, Jan Enghild*, M. Sharon Stack*, Stephen Gately*. *Division of Hematology/Oncology, Department of Obstetrics and Gynecology, Northwestern University Medical School, Chicago, IL; Department of Neuropathology, New York University Medical Center, New York, NY; Department of Pathology, Duke University, Durham, NC, USA.*

Angiostatin, a proteolytic fragment of plasminogen, inhibits angiogenesis and thereby the growth of primary and metastatic cancers. Although angiostatin has been detected in tumor bearing mice, it has not been previously described in humans. We now report the identification and characterization of an angiostatin isoform in human specimens. Angiostatin4.5 is a 52 kD protein containing kringles 1 through 4 and a portion of kringle 5 of plasminogen (Lys77-Val530), that generated in a reaction requiring plasmin catalytic activity in the presence of a free sulfhydryl donor. Western blot assays, antisera raised against the carboxy terminus of angiostatin4.5 as well as a kringle 1-3 antibody detected angiostatin4.5 in 100% of human plasma samples from normal (n = 10) and cancer patients (n = 20), malignant ovarian ascites (n = 12), and brain tumor cyst fluids (n = 1). The identity of the angiostatin4.5 was further confirmed by its removal by immunoadsorbtion of plasma with lysine-sepharose. The plasma concentration was approximately 0.1-0.2 µg/ml. Angiostatin4.5, affinity-purified from malignant ascites, was confirmed to be bioactive, inhibiting bFGF-induced vascular endothelial cell proliferation. The previously reported 38-45 kD angiostatin isoform that consists of kringles 1-4 was not detected in any of the specimens. Angiostatin4.5 is the first naturally occurring human angiostatin isoform to be identified and is present in plasma, malignant cyst fluids, and malignant ascites.

Abstract# 703 **Poster Board#/Session: 87-I**

EXPRESSION OF ANGIOPOIETIN 1, ANGIOPOIETIN 2, TIE 1 AND TIE 2 RECEPTORS IN BREAST CANCER. K. Fujikawa*, E. Presman*, C. Higgins*, Y. Inai*, L. Varticovski. *Department of Biomedical Research, St. Elizabeth's Medical Center, Tufts University School of Medicine, Boston, MA, USA.*

Several growth factors whose receptors are expressed on endothelial cells have been implicated in regulation of tumor-associated angiogenesis. The progression of many tumors, including breast cancer, is associated with enhanced angiogenesis. Tie1 and Tie2 are endothelial cell receptors of the tyrosine kinase family which regulate angiogenesis in the embryo. Angiopoietin 1 and its naturally-occurring antagonist, Angiopoietin 2, are specific ligands for Tie2 and are required for normal embryonic development of the vascular system. We and others have reported that Tie1 and Tie2 mRNA and proteins are expressed in normal adult endothelial cells and vessels associated with malignant breast lesions. We now examined the expression of Tie1, Tie2, Angiopoietin 1 and Angiopoietin 2 in breast cancer and compared mRNA levels using RT-PCR. Expression of the ligands, Angiopoietin 1 and Angiopoietin 2, and Tie receptors was increased in aggressive breast cancer lesions. In addition, we found that Tie1 and Tie2 proteins are expressed at higher levels in activated microvascular young endothelial cells than in large vessels distal to the malignant lesions. Therefore, Tie1 and Tie2 and the ligands, Angiopoietin 1 and Angiopoietin 2, may participate in progression of breast cancer by enhancing the angiogenic response to the tumor. Detection of Tie receptors and their ligands provides a novel diagnostic tool for identifying tumors with angiogenic potential.

Abstract# 704 **Poster Board#/Session: 88-I**

NEUTRALIZING MONOCLONAL ANTIBODY TO VEGFR-2 (KDR) ABROGATES BASIC FIBROBLAST GROWTH FACTOR INDUCED ANGIOGENESIS. M. Peichev*, A.J. Naiyer*, L. Witte*, D. Hicklin*, J. Bohlen*, R.L. Nachman, M.A.S. Moore, S. Rafii. *Hematology Div., Cornell Medical College, ImClone Systems, NY, NY, USA.*

Basic Fibroblast growth factor (bFGF) is a cytokine with pleiotropic effects on a variety of cells. Known bFGF functions include stimulation of the migratory chemotaxis, and proliferation of endothelial cells (EC); a process referred to as angiogenesis. To date, the exact mechanism of bFGF function in the regulation of angiogenesis is not well defined. Most *in vitro* studies have used serum-containing media to evaluate the role of bFGF in the regulation of angiogenesis. We have shown that serum contains vascular endothelial growth factor (VEGF) (PNAS. Sept 1997). Therefore, the effect of bFGF in these studies may have been mediated through modulation of VEGF receptor-2, (VEGFR-2, KDR), which is the principal specific mitogenic receptor for ECs. Given that expression of KDR is the critical limiting factor in VEGF response, we hypothesized that bFGF may regulate angiogenesis through modulation of KDR expression. Studies analyzing KDR expression have been hampered by the lack of neutralizing anti-KDR MoAbs. Therefore, in order to study regulation of KDR, we have developed high affinity as well as neutralizing MoAb to the extracellular domain of KDR. Using FITC conjugated MoAb to KDR we have demonstrated by FACS analysis, that within 8 hours of treatment of human umbilical vein endothelial cells (HUVEC) with bFGF (5 ng/ml), there is a 4.5 fold upregulation of KDR surface expression. In parallel using semi-quantitative RT-PCR we have shown that within 8 hours of incubation of HUVEC with bFGF (5 ng/ml) there is 20 fold increase in the expression of KDR mRNA. Cell cycle analysis using propidium iodide uptake, and cell proliferation as measured by ^{3}H-thymidine incorporation in HUVECs cultured in serum-free conditions demonstrated that bFGF alone does not support their survival and proliferation. However pretreatment of HUVECs with bFGF (5 ng/ml) followed by VEGF (10 ng/ml), resulted in a 1.5 fold increase in cell number, of which only a small percentage were undergoing apoptosis. In contrast, quantification of early apoptosis by Annexin V expression of the cells that were treated only with bFGF revealed that almost all the cells were in the early stages of apoptosis. Moreover, incubation of HUVECs with neutralizing anti-KDR MoAb (10 µg/ml) blocked proliferative effect of 10 ng/ml of bFGF by 75 ± 5%. In addition, MoAb to KDR completely inhibited migration of bFGF primed HUVECs through collagen coated Boyden Chambers, as well as endothelial tube formation in matrigel angiogenesis assays. These studies suggest that bFGF induce angiogenesis primarily through upregulation of KDR. Therefore anti-KDR MoAbs may be used as anti-angiogenic factors to treat malignancies that are driven by bFGF and/or VEGF.

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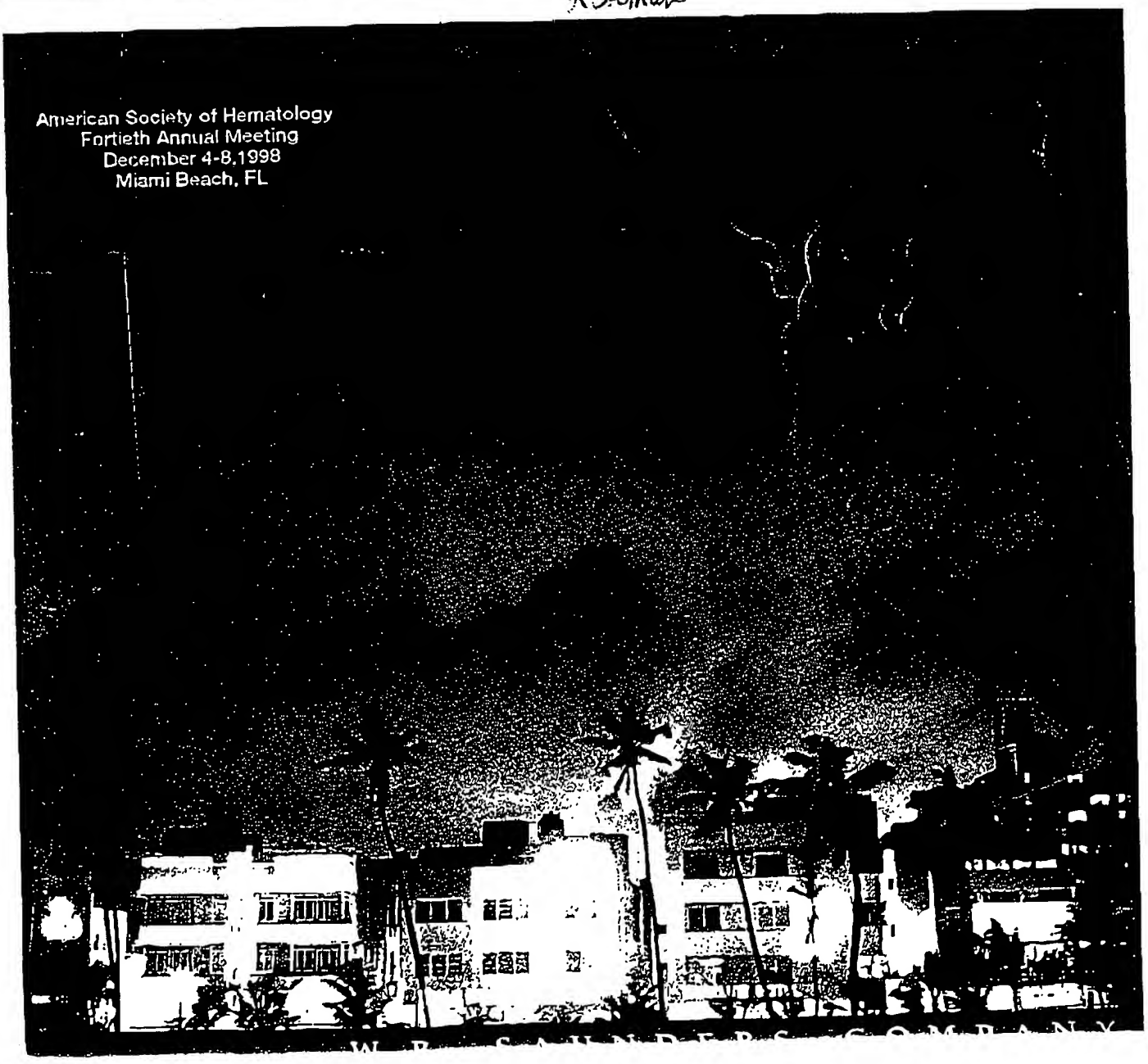

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